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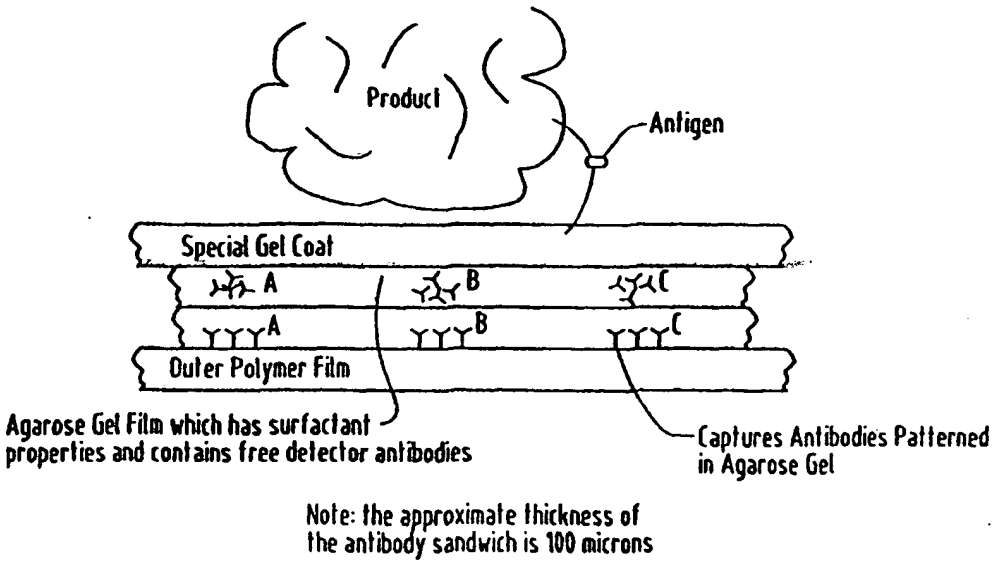

(57) Abstract

The present invention relates to bioassay materials useful for the detection of toxic substances and, more particularly, to packaging materials for food and other products, along with methods for their manufacture and use. The invention provides a unique composite material capable of detecting and identifying multiple biological materials within a single package. The biological material identification system is designed for incorporation into existing types of flexible packaging material such as polyolefin films, and its introduction into the existing packaging infrastructure will require little or no change to present systems or procedures.

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Method and Apparatus for Selective Biological Material Detection

Field of the Invention

This invention relates to the detection of pathogenic microorganisms, or biological materials, and more particularly relates to a composite bioassay material useful for the detection of particular toxic substances, its method of manufacture and method of use, wherein the composite material is particularly useful for food packaging and the like, and is capable of simultaneously detecting and identifying a multiplicity of such biological materials.

Background of the Invention

Although considerable effort and expense have been put forth in an effort to control food borne pathogenic microorganisms, there nevertheless exist significant safety problems in the supply of packaged food. For example, numerous outbreaks of food poisoning brought about by foodstuffs contaminated with strains of the E-Coli, Campylobacter, Listeria, Cyclospora and Salmonella microorganisms have caused illness and even death, not to mention a tremendous loss of revenue for food producers. These and other microorganisms can inadvertently taint food, even when reasonably careful food handling procedures are followed. The possibility of accidental contamination, for example by temperature abuse, in and of itself, is enough to warrant incorporation of safe and effective biological material diagnosis and detection procedures. Further complicating the situation is the very real possibility that a terrorist organization might target either the food or water supply of a municipality or even a nation itself, by attempting to include a

pathogenic microorganism or toxic contaminant capable of causing widespread illness or even death. If, by accident or design, the food supply of a particular population were to be contaminated, it is not only imperative that the population be alerted to the contamination, but it is further necessary that the particular contaminant be quickly and precisely pinpointed so that appropriate countermeasures may be taken.

Thus, if it were possible to readily substitute standard packaging materials with a flexible material capable of

1) quickly and easily detecting the presence, and

2) indicating the particular identity of a variety of pathogenic biological materials, a long felt need would be satisfied.

Description of the Prior Art

The Berkeley Lab Research News of 12/10/96, in an article entitle "New Sensor Provides First Instant Test for Toxic E.Coli Organism" reports on the work of Stevens and Cheng to develop sensors capable of detecting E. Coli strain 0157:H7. A color change from blue to red instantaneously signals the presence of the virulent E. Coli 0157:H7 microorganism. Prior art required test sampling and a 24 hour culture period in order to determine the presence of the E. Coli microorganism, requiring the use of a variety of diagnostic tools including dyes and microscopes. An alternative technique, involving the use of polymerase chain reaction technology, multiplies the amount of DNA present in a sample until it reaches a detectable level. This test requires several hours before results can be obtained. The Berkeley sensor is inexpensive and may be placed on a variety of materials

such as plastic, paper, or glass, e.g. within a bottle cap or container lid. Multiple copies of a single molecule are fabricated into a thin film which has a two part composite structure. The surface binds the biological material while the backbone underlying the surface is the color-changing signaling system.

The Berkeley researchers do not teach the concept of incorporating a sensor within food packaging, nor do they contemplate the inclusion of multiple sensors capable of both detecting and identifying the source of pathogenic contamination to a technically untrained end user, e.g. the food purchaser or consumer.

U.S. Patent 5,776,672 discloses a single stranded nucleic acid probe having a base sequence complementary to the gene to be detected which is immobilized onto the surface of an optical fiber and then reacted with the gene sample denatured to a single stranded form. The nucleic acid probe, hybridized with the gene is detected by electrochemical or optical detection methodology. In contrast to the instantly disclosed invention, this reference does not suggest the immobilization of the probe onto a flexible polyolefin film, nor does it suggest the utilization of gelcoats having varying porosities to act as a control or limiting agent with respect to the migration of antibodies or microbial material through the bioassay test material, or to serve as a medium for enhancement of the growth of the microbial material.

U.S. Patent 5,756,291 discloses a method of identifying oligomer sequences. The method generates aptamers which are capable of binding to serum factors and all surface molecules. Complexation of the target molecules with a mixture of nucleotides occurs under conditions wherein a complex is formed with the specific

binding sequences but not with the other members of the oligonucleotide mixture. The reference fails to suggest the immobilization of the aptamers upon a flexible polyolefin base material, nor does it suggest the use of a protective gelcoat layer which acts as a means to selectively control the migration of antibodies and antigens, or to serve as a medium for enhancement of the growth of microbial material.

Summary of the Invention

The present invention relates to packaging materials for food and other products, along with methods for their manufacture and use. The presence of undesirable biological materials in the packaged material is readily ascertained by the consumer, merchant, regulator, etc. under ordinary conditions and without the use of special equipment. A multiplicity of biological materials threaten our food supply. The present invention provides a unique composite material capable of detecting and identifying multiple biological materials within a single package. The biological material identification system is designed for incorporation into existing types of flexible packaging material such as polyolefin films, and its introduction into the existing packaging infrastructure will require little or no change to present systems or procedures. Thus, the widespread inclusion of the biological material detecting system of the instant invention will be both efficient and economical.

In one embodiment of the invention the biological material detecting system prints a pattern containing several antibodies or aptamers onto a packaging material which is usually a type of polymeric film, preferably a polyolefin film and most preferably a polyethylene film which has undergone a surface treatment, e.g. corona

discharge to enhance the film's ability to immobilize the antibodies upon its surface. The agents are protected by a special abrasion resistant gel coat in which the porosity is tailored to control the ability of certain antibodies, toxic substances, etc. to migrate therethrough. Each antibody is specific to a particular biological material and is printed having a distinctive icon shape. The detection system may contain any number of antibodies capable of detecting a variety of common toxic food microbes; although any number of microbes may be identified via the inventive concept taught herein, for the purpose of this description, the microbes of interest will be limited to E.Coli, Salmonella, Listeria and Cyclospora.

An important feature of the biological material detection system is its all-encompassing presence around and upon the product being packaged. Since the biological material detecting system is designed as an integral part of 100% of the packaging material and covers all surfaces as utilized, there is no part of the packaged product which can be exposed to undetected microbes. In the past, the use of single location or *in situ* detectors have left a majority of the area around and upon the packaged product exposed to undetected microbes. This greatly increased the chance that a spoiled or tainted product might be inadvertently consumed before the toxic agent had spread to the location of the *in situ* detector. The biological material detection system of the present invention avoids this problem by providing a plurality of individual detectors per unit area which are effective to insure positive detection of any pathogenic microorganisms within the product being tested. In order to be effective a particular degree of sensitivity is required, e.g. the detecting system must be capable of positively identifying one microbial cell in a 25 gram meat sample In a

preferred embodiment, four detectors per square inch of packaging material surface have been utilized, and in a most preferred embodiment nine or more detectors per square inch are incorporated upon the film's surface.

By use of the biological material detection system of the present invention a packager or processor can independently determine the multiplicity and identity of those biological materials against which the packaged product is to be protected. Although it is envisioned that the large majority of biological material detection treated packaging will be generic to approximately four of the most common microbes, the system will nevertheless allow each user to customize the protection offered to the public.

The biological material detecting system will not merely detect the presence of biological materials, it will also identify the particular biological materials located in a packaged product. This unique feature allows for the immediate identification of each particular biological material present since the antibodies are specific to a detector having a definitive icon shape or other identifying characteristic. Although the end use consumer is primarily interested in whether a food product is, or is not, contaminated per se, the ability to detect and identify the particular biological material immediately is of immeasurable value to merchants, processors, regulators and health officials. The ability to immediately identify a toxic material will lead to greatly reduced response times to health threats that might be caused by the biological material and will also enhance the ability for authorities to locate the source of the problem. The biological material detecting system of the present invention exhibits an active shelf life in excess of 1 year under normal operating conditions. This enhances the use of a biological material detection system

on products which are intended to be stored for long periods of time. If these products are stored so as to be ready for immediate use in some time of emergency, then it is extremely beneficial to definitely be able to determine the safety of the product at the time that it is to be used.

One particularly important feature of the biological material detecting system of the instant invention is its ability to quantitatively sensitize the reagents so as to visually identify only those biological materials which have reached a predetermined concentration or threshold level which is deemed to be harmful to humans.

For example, almost all poultry meat contain traces of the salmonella bacteria. In most cases, the salmonella levels have not reached a harmful level of concentration. The biological material detecting reagents are designed to visually report only those instances where the level of concentration of biological materials are deemed harmful by health regulatory bodies.

The method of production of the biological material detecting system is designed to be easily incorporated within the packaging infrastructure of existing systems without disruption of the systems or the procedures under which they are operating. The biological material detecting system can be incorporated onto packaging films which are produced by the packager, or those which are supplied by a film manufacturer. The apparatus necessary for applying the biological material detecting system may be easily located at the beginning of any continuous process such as printing or laminating and will operate as an integral part of an existing system.

The biological material detecting system of the instant invention represents an entirely new packaging material which is designed to inform the consumer of the presence of certain biological materials or pathogens

present in food stuffs or other materials packaged within the detecting system. The system is designed so that the presence of a biological material is presented to the consumer in a distinct, unmistakable manner which is easily visible to the naked eye. Recent outbreaks of E.Coli and other health hazards have presented serious problems to the general population and have raised concerns regarding the safety of the food supply.

It is an objective of the present invention to provide a biological material detecting system for protecting the consumer by detecting and unmistakably presenting to the untrained eye visual icons on the packaging material which signify the presence of a number of pathogens in the food stuff or other materials which are at a level harmful to humans.

It is another objective of the instant invention to provide a bioassay material wherein an antigen detecting antibody system is immobilized upon the surface of a flexible polyolefin film.

It is a further objective of the invention to provide a biological material detecting system which is so similar in appearance and utilization that its use, in lieu of traditional packaging materials, is not apparent to the food processor or other packagers.

A still further objective of the present invention is to provide a biological material detecting system which is cost effective when compared to traditional packaging materials.

Other objectives and advantages of this invention will become apparent from the following description taken in conjunction with the accompanying drawings wherein are set forth, by way of illustration and example, certain embodiments of this invention. The drawings constitute a part of this specification and include exemplary

embodiments of the present invention and illustrate various objects and features thereof.

Brief Description of the Drawing

Figure 1 is a cross-sectional interpretation of an antibody sandwich immunoassay device;

Figure 2 is a cross-sectional interpretation of a single ligand assay;

Figure 2A is a cross-sectional interpretation of a single ligand assay including a chromogenic ligand;

Figure 3 is a diagrammatic representation showing the functioning of a single ligand assay;

Figure 4 is a cross-sectional interpretation of an antibody sandwich immunoassay including a scavenger system for microbial quantification;

Figures 5 and 6 are a diagrammatic representation showing the functioning of a sandwich assay/scavenger system;

Figure 7 is a planar view of an example of icon placement and printing;

Figure 7A is an example of a typical code of identification applied to the icon pattern;

Figure 8 is the result derived from EXAMPLE 2 and exemplifies capture sensitivity of a single ligand treated polyethylene film;

Figure 9 is a block diagram of the apparatus illustrating the process steps for forming a sandwich assay;

Figure 10 is a block diagram of the apparatus illustrating the process steps for forming a single ligand assay.

Description of the Preferred Embodiment(s)

Referring now to Figure 1, the detection and identification of various biological materials in packaged foods or other products is accomplished by the use of antibodies which are specific to the biological material being sought. Specific antibodies, defined as capture antibodies, are biologically active ligands characterized by their ability to recognize an epitope of the particular toxic substance being tested for. These capture antibodies are selected from such materials as antibodies, aptamers, single stranded nucleic acid probes, lipids, natural receptors, lectins, carbohydrates and proteins. In one embodiment of the invention, the capture antibodies are arranged with unique icon shapes and in particular patterns. The capture antibodies are immobilized to the polymer film. An agarose gel coat containing detector antibodies is printed in register above the capture antibodies. A protective gel coat completes the construction of the packaging material. The gel coat constituting the inner layer, e.g. that layer which is next to the packaged product, is a special type of gel coat or an equivalent thereto which has sufficient porosity to allow toxic molecules, known as antigens, to migrate through it to an antibody "sandwich" laminated between the polymer film and the gel coat. The special gel coat has sufficient abrasion resistance to prevent exposure of the reagents to the product. The special gel coat useful in the invention is a readily available coating commonly utilized in the food industry to coat candies and the like, e.g. coated chocolates to prevent them from melting on one's hands. Migration of antigens is driven by capillary action and normally reaches a state of equilibrium within a 72 hour time period. In a

particularly preferred embodiment, when operating within a temperature range of 4 - 25 degrees Celsius, an initial positive reading can be obtained within 30 minutes, and the test continues to yield results for about 72 hours. Upon migrating through the special gel coat the antigen enters an agarose gel film which has surfactant properties, contains free detector antibodies, and also contains ingredients designed to enhance the growth of microbial materials, e.g. nutrients such as sorbitol, NOVOBIOCIN, CEFIXIME and TELLURITE which increase the growth rate and ease isolation of E. Coli 0157H. If the antigen encounters a species of antibody which is specific to an epitope thereof, it will then bind to it forming a detector/antibody complex. Once bound thereto, the bound antigen/antibody complex becomes too large to migrate back through the special gel coat due to its inherent fine porous structure. This insures that pathogenic material can not migrate back into the product being tested. Continuing pressure toward equilibrium from capillarity will tend to move the antigen, with its bound antibody, through a second gel coat layer and into an area of the flexible polyolefin film containing corresponding species of immobilized capture antibodies. The layer of immobilized antibodies is attached to the outer polymer film in predetermined patterns of simple icons, as best seen in Figures 7,7A. When the particular species of bound antigen encounters a particular corresponding species of immobilized antibody specific to a separate and distinct epitope thereof, further binding occurs. Upon the antigen binding to the two antibodies, a distinct icon shape emerges on the outer film at the point of binding, thereby providing a visual indicator.

While it is theoretically possible to detect an unlimited number of pathogens present in a packaged product, then to present this information in a very clear and unmistakable manner to an untrained consumer, as a practical matter there are limits to the amount of information which can be developed and presented in the biological material detecting system. Some of the limiting factors are cost, available surface area for display of information, complexity, and other considerations. Thus, for illustrative purposes only, the biological material detecting system as exemplified herein utilizes four separate pairs of antibodies, as set forth in Figures 7 and 7A. This is in no way meant to suggest a limit on the number of antibodies that can be utilized in a single biological material detecting system.

As demonstrated in Figures 7 and 7A, the invention is exemplified with reference to detection of the following four microbes:

1. E-Coli;
2. Salmonella;
3. Listeria; and
4. Cyclospora.

To each of the four microbes, a particular icon shape is assigned. Although there are infinite numbers of icons which might be used including letters, numbers, or even words, we have chosen simple identifiers for the purpose of demonstration. As an initial step in the construction of the biological material detecting system, the outer polymer film or base layer undergoes a printing process in which a pattern of the four icons, wherein each icon utilizes a specific species of immobilized capture antibody, is applied thereto. Corresponding species of free antibodies, known as detector antibodies, which are

biologically active ligands characterized by their ability to recognize a different epitope of the same particular toxic substance being tested for, and suspended in an agarose gel solution containing a surfactant and a nutrient, are printed in registration with the immobilized antibodies so as to be in overlying and juxtaposed relationship thereto, and are then dried. Lastly, a second gel coat having a degree of porosity sufficient to prevent passage of the detector antibodies is laminated to the preparation.

Although the detection of biological materials through the use of antibodies is well known, there are several new and novel aspects to the application of antibody science which are set forth in the development of the biological material detecting system of the present invention.

Among these are: 1) the use of multiple antibodies to detect multiple biological materials in individual packages; 2) the use of a distinctive icon or other shape to not only detect, but visually identify the biological materials to the consumer, vendor, regulator, etc.; 3)insuring that detection and identification of the biological materials is accomplished in a timely manner in each particular application by judiciously controlling the porosity of the gel coat, thereby controlling the lapse rate of the reaction through the strength of capillary action; 4) inclusion of additives within the special gel coat to enhance the levels of microbes present; 5) incorporating the biological material detecting system of the instant invention within the existing packaging industry infrastructure; and 6) providing a bioassay material and methods for its production and use which immobilizes the antibodies onto the surface of a flexible

polyolefin, e.g. a surface treated polyethylene, polypropylene or mixture thereof.

The embodiment discussed above is based upon a sandwich immunoassay as depicted in Figure 1, which measures specific microbes, wherein the particular toxic substance is one or more members selected from the group consisting of a particular microorganism, biological materials containing the genetic characteristics of said particular microorganism, and mutations thereof. In a particular embodiment, the toxic substance is selected from the group consisting of microorganisms, nucleic acids, proteins, integral components of microorganisms and combinations thereof.

It should also be understood that the invention will function by direct measurement of microbes with certain types of antibodies, selected from the group consisting of an antibody, a single stranded nucleic acid probe, an aptamer, a lipid, a natural receptor, a lectin, a carbohydrate and a protein. The biological materials may also be measured by non-immunological methods in particular using labeled molecules, such as aptamers, which have a high affinity for the biological materials.

The invention utilizes various types of detector antibodies, e.g. those conjugated with dyes to produce a visual cue, or alternatively, photoactive compounds capable of producing a visual cue in response to a particular type of light exposure, for example a scanning system which detects luminescent properties which are visualized upon binding of the antigen and antibody. In this method of construction biological materials are measured directly with a biologically active ligand, e.g. an antibody, aptamer, nucleic acid probe or the like,

which induces a conformational change to produce a visual cue.

It is also understood that specific polymers may be incorporated into the invention and that when a biological material is bound to the surface it induces a molecular change in the polymer resulting in a distinctly colored icon. Referring to Figures 2 and 2A, in an alternative embodiment a sandwich-type of construction is not necessary. As depicted in Figures 2 and 2A, the provision of certain types of biologically active ligand, e.g. chromogenic ligands to which receptors are bound will permit the visual confirmation of binding of the antigen to the immobilized ligand.

As depicted in Figure 3, a polymer film is provided and a biologically active ligand, preferably a chromogenic ligand, is immobilized to the polymer film. In the past, immobilized ligands were attached to rigid solid support matrices such as plastic, polystyrene beads, microtitre plates, latex beads, fibers, metal and glass surfaces and the like. The immobilized ligands have also been attached to flexible surfaces such as nitrocellulose or polyester sheets which were not transparent. Surprisingly, the inventor has discovered that it is possible to attach biologically active ligands to the surface of a polyolefin sheet having appropriate properties of transparency and flexibility and that the composite functions as a biological sensor or assay material. After printing on the reactive polymer film, the material goes through a drying step; subsequent to which a special gel coat or liquid film is applied as a protectant layer and the final product is then dried.

Illustrative of films which will function in the present invention is a film containing a structural polymer base having a treated surface and incorporating therein a fluorescing antibody receptor and finally a stabilized gel coat. These films are created by first exposing the film to an electron discharge treatment at the surface thereof, then printing with a fluorescing antibody receptor. Subsequently, a drying or heating step treats the film to immobilize the receptor. Next, the film is washed to remove un-immobilized receptor; the film is then coated with a gel and finally dried. Examples of the types of commercially available films which might be utilized are a straight polyethylene film with electron discharge treatment marketed under the trademark SCLAIR®. The electron discharge treatment renders the film much more susceptible to immobilization of the antibodies on its surface. Additional films which might be utilized are Nylon 66 films, for example DARTEK®, a coextrudable adhesive film such as BYNEL® and a blend of BYNEL® with polyethylene film.

With reference to Figures 4-6, one of the most important features of the biological material detecting system is its ability to quantitatively sensitize the antibody or aptamer so as to visually identify only those biological materials that have reached a concentration level deemed harmful to humans. One means of providing this sensitization is by including a scavenger antibody which is a biologically active ligand characterized as having a higher affinity for the particular toxic substance than the capture antibody. The scavenger antibody is provided in a sufficient amount to bind with the particular toxic substance up to and including a specific threshold concentration. In this manner, the

capture antibody will be prevented from binding with a detector antibody until the concentration of the particular biological material surpasses the specific threshold concentration. In this manner, the biological material detecting system visually reports only those instances where concentration levels are deemed harmful by health regulatory bodies.

Since the biological material detecting system as described herein can maintain its activity over long periods of time, e.g. up to 1 year, it is able to protect against contamination in products which have long shelf lives. Additionally, by reporting only toxic concentrations, it avoids "false positives" and, in some cases, can extend the useful life of the product.

Referring to Figures 9 and 10, the apparatus for producing the biological material detecting system is illustrated. These embodiments are essentially particular combinations of printers, coaters and dryers which will be used to place biologically active reagents upon a thin polymer film useful for packaging food stuffs and other products. These films will be further processed subsequent to application of the biological material detecting system by printing, laminating, or equivalent methods of fabrication. The machinery is designed so that it will transport and process very thin films at rather high speeds. Furthermore, the machinery is designed so that it can be utilized effectively as an additional processing step when added to continuous processing operations already in use at packaging material fabrication plants. The printing machinery is designed so that a minimum of four distinct biological active ligands in a hydrate solution can be printed in patterns in a precise registration on the polymer film. The printing

may be accomplished by jet spray or roller application, or equivalent printing methods. Each print applicator is capable of printing a detailed icon no larger than 1/4" x 1/4" in a minimum thickness. Patterning may be controlled by computer or roller calendaring. It is important to determine the appropriate viscosity of the solution to be applied so that successful printing, coating, and drying can be accomplished. After the printing step the icons must be protected. This is accomplished by a final application of a thin special gel coat or a thin liquid film. This step is accomplished by a 100% coating of the entire film or alternatively by selectively coating each icon such that a 10% overlap is coated beyond the icon in all directions. This coating step may be accomplished with sprays or rollers and the viscosity of the coating material must be optimized so as to provide adequate coverage. The biological material detecting system must be dried after printing and once again after coating. The drying is accomplished in a very rapid manner so as to enable high through put for the process. Various means of drying include the use of radiant heat, convected air and freeze drying. Care must be taken to avoid drying temperatures which will inactivate the biological reagents which have been applied. The polymer film which has been surface treated in the form of electron discharge, e.g. corona treatment, is most preferred. After preparation, the thin film is transported at relatively high speeds so that a wrinkle free surface is provided for printing, coating and rollup. Additionally, the apparatus provides a complete recovery system for the reagents which allows for total recovery of the agents and the volatile organic contaminants.

The invention will be further illustrated by way of the following examples:

EXAMPLE 1

Detection of Antibody on the Surface of a Pre-Treated Thin Layer Polyethylene Sheet:

Rabbit polyclonal IgG was diluted to a final concentration of 2.0 μg/ml in 0.1M carbonate (Na_2CO_3)-bicarbonate ($NaHCO_3$) buffer, pH 9.6.

Using a 2" x 3" grid, 75 μL (150 ng) was applied to a sheet of pre-treated polyethylene at 1"intervals.

The antibody treated polyethylene sheet was dried for 1.5 hrs. at a temperature of 37°C.

The dried sheet was then washed 3 times with a phosphate buffered saline solution at a ph of 7.4.

HRP conjugated goat anti-rabbit IgG ($G\alpha R^{HRP}$) was diluted to a concentration of 1:7000 in 1% casein, 0.1M potassium ferricyanide $K_3Fe(CN)_6$, 0.1% phosphate glass ($Na_{15}P_{13}O_{40}$ - $Na_{20}P_{18}O_{55}$), at a pH of 7.4.

A precision pipette was used to apply 125 μL of diluted G^{HRP} to the grid backed polyethylene sheet at 1" intervals coinciding with the area covered by the previously couples $R\alpha G$.

The sheet was incubated at room temperature for 30 minutes.

The sheet was then washed 3X with phosphate buffered saline at a pH of 7.4.

125μL of precipitating TMB enzyme substrate was added to the test areas.

The sheet was incubated at room temperature until color development was complete.

Lastly the sheet was washed 3 times with deionized water and allowed to air dry.

EXAMPLE 2

Full Sandwich Immunoassay on the Surface of a Pre-Treated Thin Layer Polyethylene Sheet

Rabbit polyclonal IgG was diluted to a final concentration of 2.0 μg/ml in 0.1M carbonate (Na_2CO_3)-bicarbonate ($NaHCO_3$) buffer, pH 9.6.

A 13 x 9 cm piece of pre-treated thin layered polyethylene sheet available from Dupont was inserted into a BIO-RAD DOT-SPOT apparatus possessing 96 sample wells spaced at 1.0 cm intervals in a 12 x 8 well grid.

A 100 μL sample (1.0 μg) of rabbit polyclonal IgG was applied to each well 8 of column 1.

Antibody samples applied to columns 2-12 represented serial dilutions of the antibody ranging from 500 ng - 0.5 ng.

The antibody treated polyethylene sheet was dried overnight at 37° C.

The dried sheet was washed 3 times with phosphate buffered saline (PBS), pH 7.4.

Antigen was diluted to a final concentration of 1.0 μg/ml in tris buffered saline (TBS) with 1% casein, pH 7.4.

100 μL, representing 100 ng, of antigen, was applied to each well of the apparatus and incubated at room temperature for 1 hour.

The polyethylene sheet was washed 3 times with phosphate buffered saline (PBS), pH 7.4.

Detector mouse monoclonal antibody was diluted was diluted 1:625 with TBS containing 1% casein, 0.1M potassium ferricyanide $K_3Fe(Cn)_6$, and 0.1% phosphate glass ($Na_{15}P_{13}O_{40}$ - $Na_{20}P_{18}O_{55}$), pH 7.4.

100 μL of the 1:625 dilution of detector antibody solution was applied to each well of row # 1.

Detector samples of 100 μL applied to rows 2-7 represented serial dilutions of the antibody ranging from 1:1,250 to 1:80,000. Dilutions of detector antibody were incubated on the polyethylene sheet for 1 Hr. at room temperature.

The polyethylene sheet was washed 3 times with phosphate buffered saline (PBS), pH 7.4.

100 μL of goat anti-mouse IgG^{HRP} were added to each well of the DOT-SPOT apparatus and allowed to incubate for one hour at room temperature.

The polyethylene sheet was washed 3 times with phosphate buffered saline (PBS), pH 7.4.

100 μL of precipitating TMB enzyme substrate was added to the test areas.

The sheet was incubated at room temperature until color development was complete (see Figure 8).

Lastly the sheet was washed 3 times with deionized water and allowed to air dry.

It is to be understood that while a certain form of the invention is illustrated, it is not to be limited to the specific form or arrangement of parts herein described and shown. It will be apparent to those skilled in the art that various changes may be made without departing from the scope of the invention and the invention is not to be considered limited to what is shown in the drawings and described in the specification.

I CLAIM:

Claim 1. A biological assay material for detecting the presence of a particular toxic substance comprising:

a base layer which is flexible polyolefin film having a surface which has undergone a treatment step effective to enhance said film's ability to immobilize a ligand applied thereto;

a capture antibody which is a biologically active ligand characterized by its ability to recognize an epitope of the particular toxic substance, said ligand being immobilized onto said surface of said polyolefin film;

a first agarose gelcoat layer overlying the capture antibody, said agarose layer being permeable to the toxic substance and containing ingredients to enhance the growth thereof, said layer further containing a detector antibody which is a biologically active ligand characterized by its ability to recognize a different epitope of said particular toxic substance, thereby forming a detector antibody/antigen complex; and

a second protective gelcoat layer overlying the detector antibody and having a degree of porosity whereby passage of said toxic substance is permitted and passage of said detector antibody/antigen complex is prevented, said second protective gelcoat layer having a degree of abrasion resistance effective to protect the biological assay material.

Claim 2. The biological assay material according to claim 1 wherein the flexible polyolefin film is selected from the group consisting of polyethylene, polypropylene and mixtures thereof.

Claim 3. The biological assay material according to claim 1 wherein the polyolefin film is surface treated by a corona discharge process.

Claim 4. The biological assay material according to claim 1 wherein the particular toxic substance is one or more members selected from the group consisting of a particular microorganism, biological materials containing the genetic characteristics of said particular microorganism, and mutations thereof.

Claim 5. The biological assay of material according to claim 1 wherein the particular toxic substance is selected from the group consisting of microorganisms, nucleic acids, proteins, integral components of microorganisms and combinations thereof.

Claim 6. The biological assay material according to claim 1 wherein the ligand is selected from the group consisting of an antibody, a single stranded nucleic acid probe, an aptamer, a lipid, a natural receptor, a lectin, a carbohydrate and a protein.

Claim 7. The biological assay material according to claim 1 further including a scavenger antibody which is a biologically active ligand characterized as having a higher affinity for the particular toxic substance than the capture antibody, said scavenger antibody being present in a sufficient amount to bind with the particular toxic substance up to and including a specific threshold concentration;

whereby a capture antibody will be prevented from binding with a detector antibody until the concentration of the particular biological material surpasses the specific threshold concentration.

Claim 8. A method to detect the presence or absence of a particular toxic substance, which method comprises:

a) providing a base layer which is a flexible polyolefin film having a surface which has undergone a treatment step effective to enhance said film's ability to immobilize a ligand applied thereto;

b) providing a capture antibody which is a biologically active ligand characterized by its ability to recognize an epitope of the particular toxic substance, said ligand being immobilized onto said surface of said polyolefin film;

c) providing a first agarose gelcoat layer overlying the capture antibody, said agarose layer being permeable to the toxic substance and containing ingredients to enhance the growth of the toxic substance, said layer further containing a detector antibody which is a biologically active ligand characterized by its ability to recognize a different epitope of said particular toxic substance;

d) providing a second protective gelcoat layer overlying the detector antibody and having a degree of porosity sufficient to prevent passage of said detector antibody therethrough;

e) placing said biological assay material in an environment which may contain a particular toxic substance; and

f) monitoring said biological assay material for a period of time sufficient to observe a visual signal which will confirm the presence or absence of the particular toxic substance.

Claim 9. A material useful for food packaging and characterized by its ability to detect the presence and particularly identify one or more toxic substances comprising:

a base layer which is a flexible polyolefin film having a surface which has undergone a treatment step effective to enhance said film's ability to immobilize a ligand applied thereto;

a capture antibody which is a biologically active ligand characterized by its ability to recognize an epitope of the particular toxic substance, said ligand being immobilized onto said surface of said polyolefin film;

a first protective agarose gelcoat layer overlying the capture antibody, said agarose layer being permeable to the toxic substance;

a detector antibody which is a biologically active ligand characterized by its ability to recognize a different epitope of said particular toxic substance, said detector antibody overlying said first protective gelcoat layer; and

a second gelcoat layer overlying the detector antibody and having a degree of porosity sufficient to prevent passage of said detector antibody therethrough.

Claim 10. The material according to claim 9 wherein the flexible polyolefin film is selected from the group consisting of polyethylene, polypropylene and mixtures thereof.

Claim 11. The material according to claim 9 wherein the polyolefin film is surface treated by a corona discharge process.

Claim 12. The material according to claim 9 wherein the particular toxic substance is one or more members selected from the group consisting of a particular microorganism, biological materials containing the genetic characteristics of said particular microorganism, and mutations thereof.

Claim 13. The material according to claim 9 wherein the particular toxic substance is selected from the group consisting of microorganisms, nucleic acids, proteins, integral components of microorganisms and combinations thereof.

Claim 14. The material according to claim 9 wherein the ligand is selected from the group consisting of an antibody, a single stranded nucleic acid probe, an aptamer, a lipid, a natural receptor, a lectin, a carbohydrate and a protein.

Claim 15. The material according to claim 9 further including a scavenger antibody which is a biologically active ligand characterized as having a higher affinity for the particular toxic substance than the capture antibody, said scavenger antibody being present in a

sufficient amount to bind with the particular toxic substance up to and including a specific threshold concentration;

whereby a capture antibody will be prevented from binding with a detector antibody until the concentration of the particular biological material surpasses the specific threshold concentration.

Claim 16. The material according to claim 9 wherein

one or more species of capture antibody are immobilized onto said surface of said polyolefin film in a particular orientation, each of said one or more species being characterized by a unique shape; and

one or more corresponding species of detector antibody are applied onto the surface of said first protective gelcoat layer in the same particular orientation as said one or more species of capture antibody, each of said one or more species being characterized by a corresponding unique shape;

whereby simultaneous binding of any of the one or more species of capture antibodies and one or more corresponding species of detector antibodies with the particular toxic substance which they recognize results in the appearance of a visual signal having the unique shape assigned to that species;

wherein an observer is alerted to the presence and identity of said particular toxic substance.

Claim 17. A biological assay material for detecting the presence of a particular toxic substance comprising:

a base layer which is a flexible polyolefin film having a surface which has undergone a treatment step effective to enhance said film's ability to immobilize a

ligand applied thereto;

a biologically active ligand immobilized to the film; and

a gel coat or liquid film applied as a protectant layer;

whereby binding of the particular toxic substance and biologically active ligand produces a visual signal which is indicative of both the presence and identity of said particular toxic substance.

Claim 18. The biological assay material according to claim 17 wherein the biologically active ligand is a chromogenic ligand.

Claim 19. The biological assay material according to claim 17 wherein the base layer is a polyolefin film incorporating thereon a fluorescing antibody receptor.

Claim 20. The biological assay material according to claim 19 wherein the base layer is created by exposing the film to an electron discharge treatment at the surface thereof, printing with a fluorescing antibody receptor and drying or heating the film to immobilize said receptor.

Claim 21. The biological assay material according to claim 17 wherein a scavenger antibody which is a biologically active ligand characterized as having a higher affinity for the particular toxic substance than the immobilized ligand is provided in a sufficient amount to bind with the particular toxic substance up to and including a specific threshold concentration;

whereby the assay material is quantitatively sensitized so as to visually identify only those particular toxic substances that have reached a concentration level deemed harmful to humans.

Claim 22. The biological assay material according to claim 18 wherein the chromogenic ligand is selected from the group consisting of those conjugated with dyes to produce a visual cue and those characterized as photoactive compounds capable of producing a visual cue in response to a particular type of light exposure;

whereby binding of the particular toxic substance and chromogenic ligand results in a color change or visualization of a luminescent property which is indicative of both the presence and identity of said particular toxic substance.

Claim 23. The biological assay material according to claim 17 wherein the material is a food packaging material.

Claim 24. The biological assay material according to claim 17 containing a plurality of biologically active ligands, each of said ligands being receptive to an epitope of a different particular toxic substance and having a unique shape;

whereby upon binding with one or more of said different particular toxic substances, a visual signal will result thereby alerting an observer to the presence and identity of any or all of the particular toxic substance to which said material is receptive.

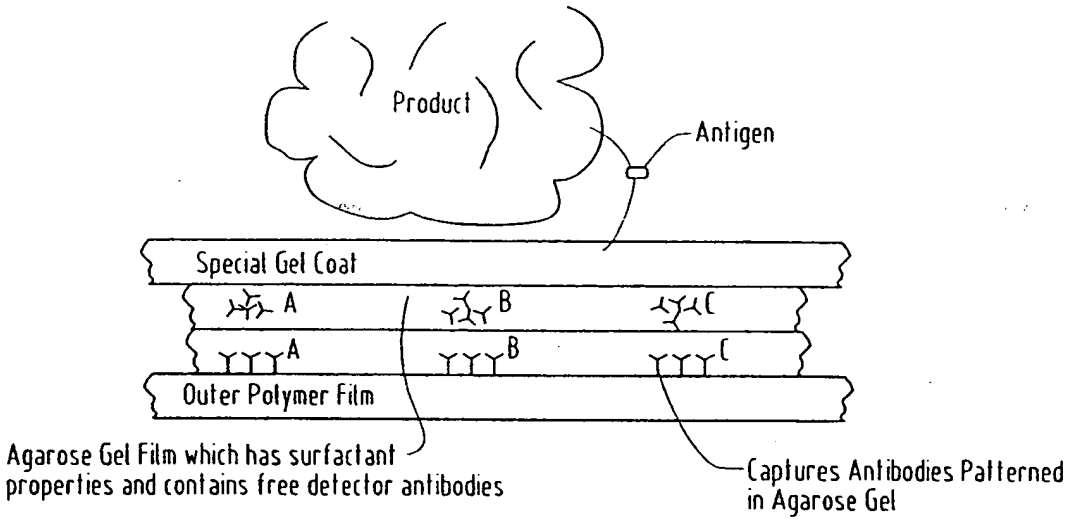
Claim 25. The biological assay material according to claim 17 wherein the particular toxic substance is one or more members selected from the group consisting of a particular microorganism, biological materials containing the genetic characteristics of said particular microorganism, and mutations thereof.

Claim 26. The biological assay of material according to claim 17 wherein the particular toxic substance is selected from the group consisting of microorganisms, nucleic acids, proteins, integral components of microorganisms and combinations thereof.

Claim 27. The biological assay material according to claim 17 wherein the ligand is selected from the group consisting of an antibody, a single stranded nucleic acid probe, an aptamer, a lipid, a natural receptor, a lectin, a carbohydrate and a protein.

Claim 28. The material according to claim 17 wherein the flexible polyolefin film is selected from the group consisting of polyethylene, polypropylene and mixtures thereof.

FIG. 1



Note: the approximate thickness of the antibody sandwich is 100 microns

FIG. 2

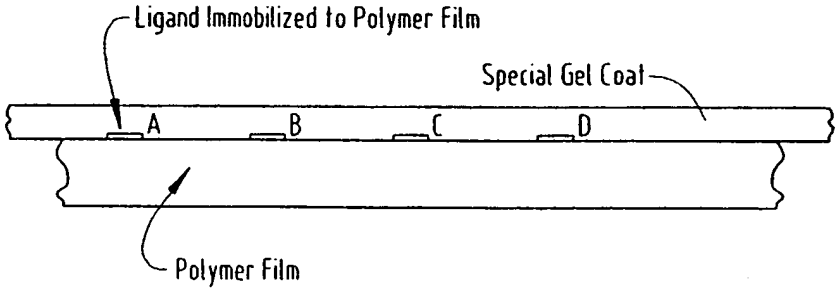
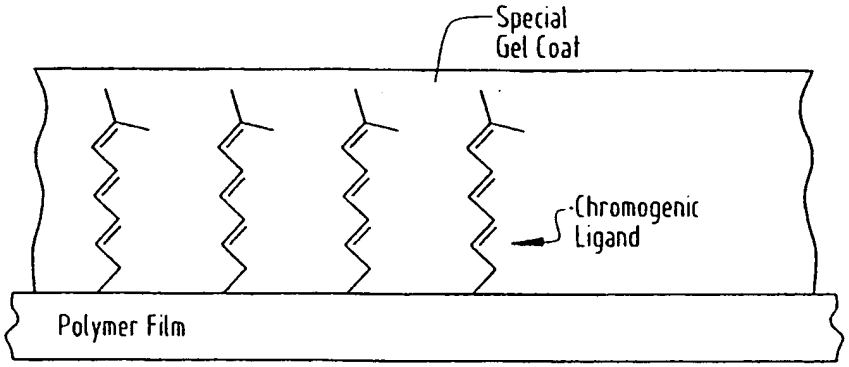


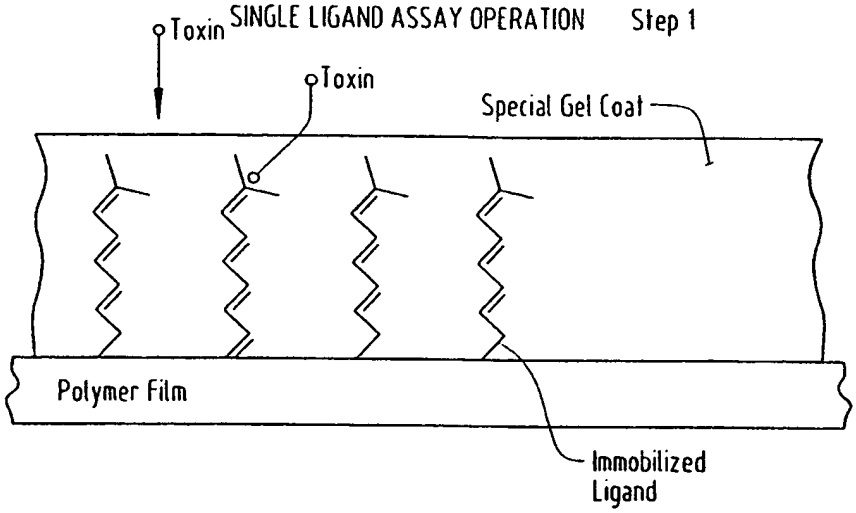
FIG. 2A

SINGLE LIGAND ASSAY CONSTRUCTION



A chromogenic ligand is immobilized on the polymer film in patterns of icons, and is coated with a pourous gel which will allow the migration of toxins to the ligand.

FIG. 3



When a toxin enters the special gel and binds to the ligand, it will cause a confirmational change in the ligand which results in a color change. Distinct patterns will emerge in about 30 minutes and distinct dark color changes will appear in 72 hours.

FIG. 4

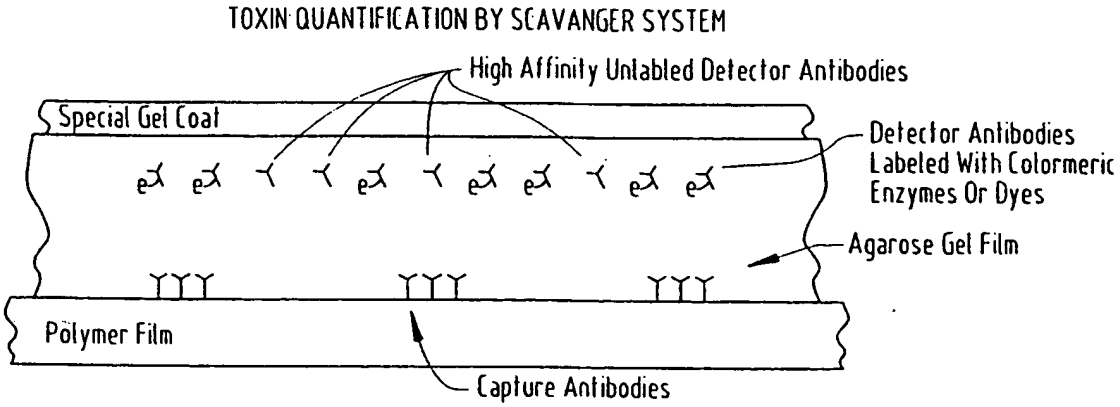
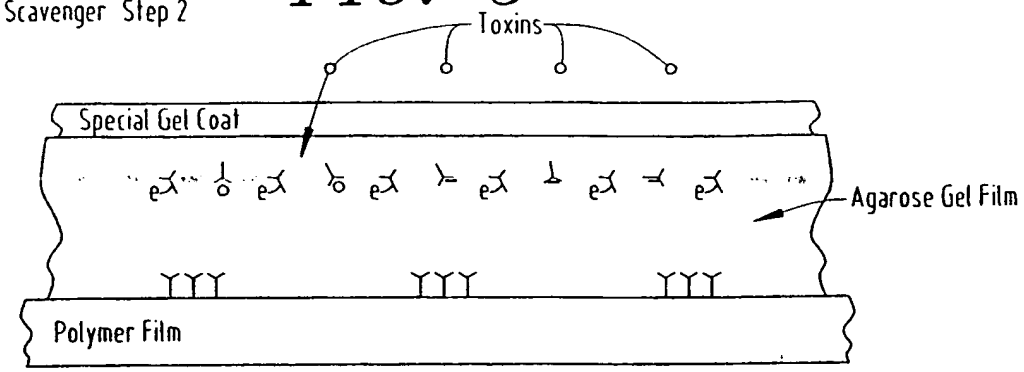


FIG. 5

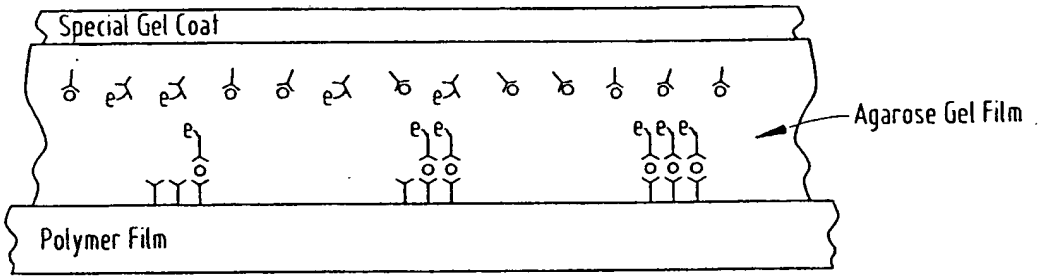


When toxins enter the sandwich, they will bind first with the unlabeled detector antibodies until all of these are bound.

FIG. 6

Scavenger Step 3

After all of the high affinity unlabeled detector antibodies are bound to the toxins, the detector antibodies labeled with a colormeric enzyme will begin to bind to the toxins. The labeled complex will then begin to bind to the capture antibodies, producing a visual cue.



after 30 minutes
distinct pattern

after 72 hours
dark distinct pattern

FIG. 7

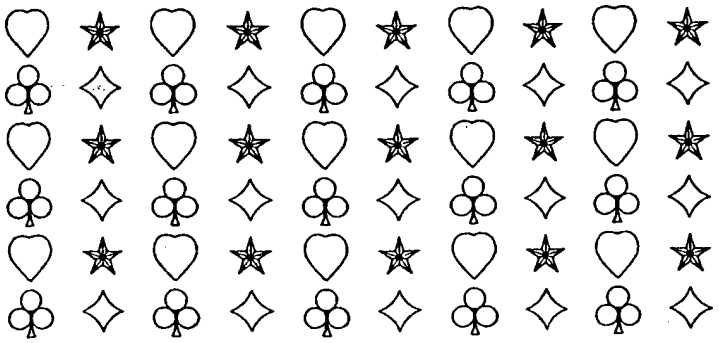


FIG. 7A

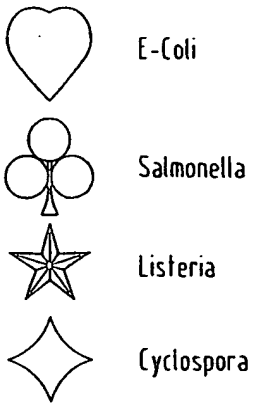


FIG. 8

Checkerboard Dot-Spot Application of RaMBP on a Polyethylene Surface and Detection by GaR^{HRP}

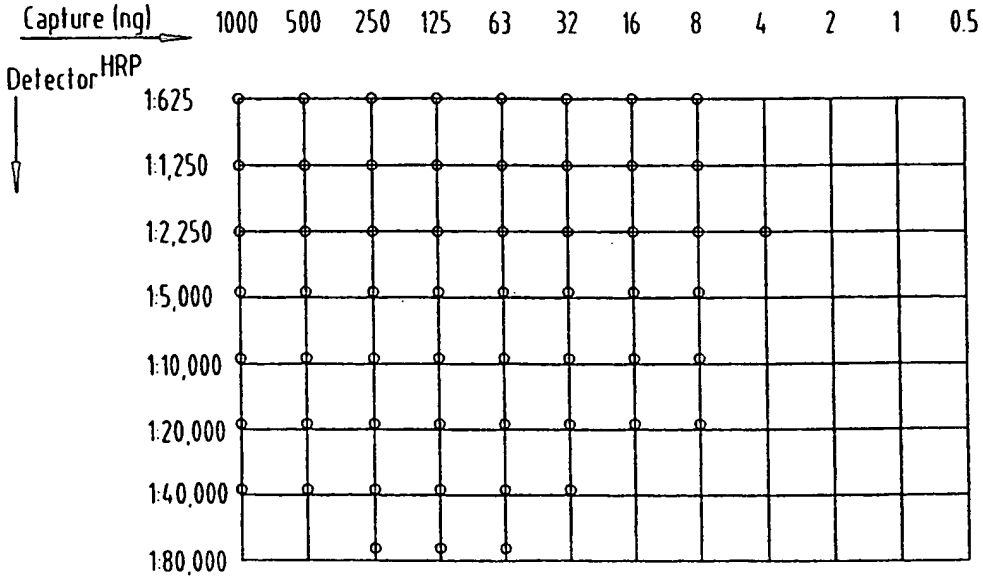


FIG. 9

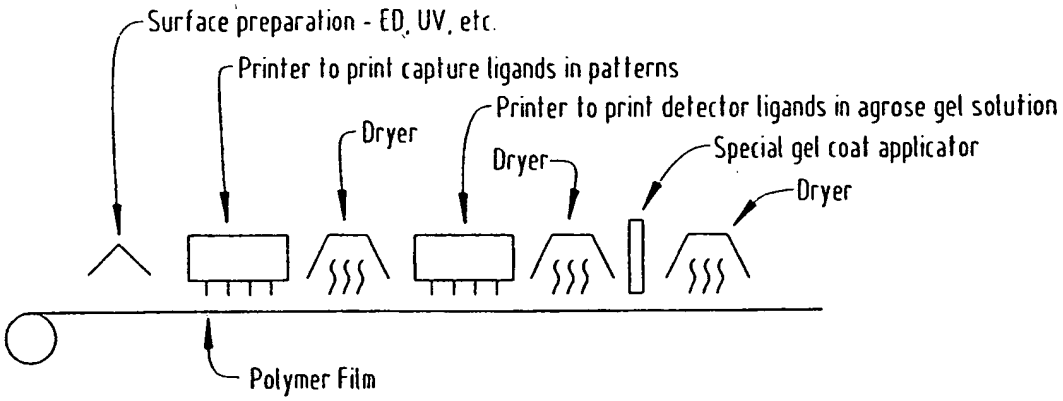
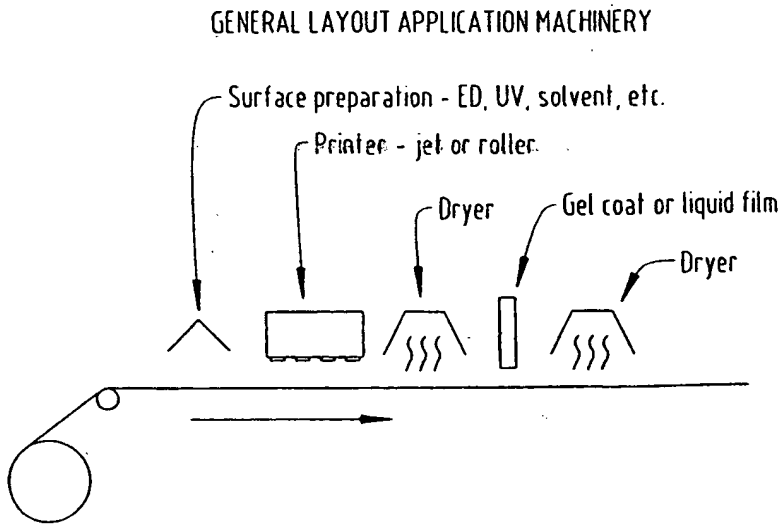

Surface preparation - ED, UV, etc.
Printer to print capture ligands in patterns
Dryer
Printer to print detector ligands in agrose gel solution
Dryer
Special gel coat applicator
Dryer
Polymer Film

FIG. 10


GENERAL LAYOUT APPLICATION MACHINERY
Surface preparation - ED, UV, solvent, etc.
Printer - jet or roller
Dryer
Gel coat or liquid film
Dryer

INTERNATIONAL SEARCH REPORT

Inte .ional Application No
PCT/IB 99/02123

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/02 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 870 005 A (AKIYOSHI YUTAKA ET AL) 26 September 1989 (1989-09-26)	17-28
A	column 1, line 28-43 column 4, line 65-69 column 5, line 1 column 8, line 50-63 column 9, line 55-68 column 10, line 1-13 examples 1-3	1-16
X	EP 0 347 839 A (FUJIREBIO KK ;FUJI PHOTO FILM CO LTD (JP)) 27 December 1989 (1989-12-27)	17-28
A	abstract page 9	1-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 2000	09/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pellegrini, P

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INTERNATIONAL SEARCH REPORT

Inte. ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 327 918 A (KONISHIROKU PHOTO IND) 16 August 1989 (1989-08-16)	17-28
A	abstract page 10-11 page 12, line 21-44 page 13-14 claims	1-16
X	US 5 266 460 A (SUDO YUKIO ET AL) 30 November 1993 (1993-11-30)	17-28
A	abstract example 1	1-16
X	US 4 857 102 A (DENNIS NICHOLAS ET AL) 15 August 1989 (1989-08-15)	17-28
A	column 6, line 16-32 example 1	1-16
A	EP 0 313 858 A (FUJI PHOTO FILM CO LTD) 3 May 1989 (1989-05-03) page 21-22	1-28
A	US 4 966 856 A (ITO TSUKASA ET AL) 30 October 1990 (1990-10-30) example 1	1-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. ional Application No
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